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Induction of a variety of tumors by c-*erbB2* and clonal nature of lymphomas even with the mutated gene (Val^{659} → Glu^{659})

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The c-*erbB2* gene is expressed uniquely in fetal epithelium *in vivo* and has been suggested to contribute to the development and/or progression of adenocarcinomas in man. In order to assess the oncogenicity of the c-*erbB2* gene *in vivo*, normal c-*erbB2* and mutant c-*erbB2* encoding glutamic acid instead of valine at position 659 within the transmembrane domain were introduced into mice under the transcriptional regulatory unit of mouse mammary tumor virus long terminal repeat (MMTV-LTR) or immunoglobulin enhancer−SV40 early gene promoter (Ig/Tp). In transgenic mice with normal c-*erbB2* under MMTV-LTR, not only adenocarcinomas but also a variety of tumors including B lymphomas were induced at relatively late onset. Induction of pre-B cell lymphomas with normal c-*erbB2* was also observed using the Ig/Tp regulatory unit within 6−10 months in some members of one transgenic family among seven lines established. In contrast, with the mutant c-*erbB2* under the Ig/Tp regulatory unit, the lymphoma was induced neonatally in all members of four transgenic families among ten lines obtained. However, the immunoglobulin heavy chain gene rearrangement pattern indicated that even with the mutant c-*erbB2* the induced lymphomas were clonal.

Key words: adenocarcinoma/c-*erbB2*/lymphoma/*neu*/transgenic mouse

Introduction

The c-*erbB2* gene was originally identified as a homolog of the c-*erbB*/epidermal growth factor (EGF) receptor gene. It encodes a 185 kd protein with tyrosine kinase activity which is supposed to function as a cell surface receptor of yet unknown external stimuli (Coussens *et al.*, 1985; King *et al.*, 1985; Semba *et al.*, 1985; Yamamoto *et al.*, 1986; Akiyama *et al.*, 1986; Stern and Kamps, 1988; Yarden and Weinberg, 1989). The gene is not expressed in hematopoietic cells but is uniquely expressed in fetal epithelium such as transitional cells of the renal pelvis and ureter, glandular cells of the gastrointestinal tract and stratified epithelium of the esophagus (Gullick *et al.*, 1987; Kokai *et al.*, 1987; Mori *et al.*, 1989). Thus, the gene product may play an important role in the growth and/or differentiation of fetal epithelial cells.

The c-*erbB2* gene is frequently amplified and over-expressed in human adenocarcinomas, especially in breast and stomach cancers, but not in other tumors such as hematologic cancers, squamous cell carcinomas or sarcomas (Yokota *et al.*, 1986, 1988; Van de Vijver *et al.*, 1987 Masuda *et al.*, 1987; Ali *et al.*, 1988; Juerin *et al.*, 1988; Tal *et al.*, 1988). Patients having breast cancers with c-*erbB2* amplification frequently relapse in the short term, do not convalesce satisfactorily, progress rapidly and have accompanying metastsis to the lymphoid organs (Slamon *et al.*, 1987; Varley *et al.*, 1987; Venter *et al.*, 1987; Zhou *et al.*, 1987; Berger *et al.*, 1988). Since amplification of c-*erbB2* is more apparent in progressed tumors, a high level of c-*erbB2* expression may contribute more to tumor progression than to development.

In a chemically transformed neuroblastoma cell line, a rat homolog of c-*erbB2* (*neu*) is activated by a point mutation which results in a single amino acid substitution (valine to glutamic acid) in the transmembrane domain of the protein (Schechter *et al.*, 1984; Bargmann *et al.*, 1986a,b). Substitution of the corresponding amino acid in human c-*erbB2* protein requires two mutations in the gene. The mutant *neu* gene, but not the normal *neu* gene, can transform NIH3T3 cells (Bargmann *et al.*, 1986b). In contrast, the human c-*erbB2* gene can transform the fibroblasts by over-expression (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987; and our unpublished data). The difference in the transforming ability of normal c-*erbB2*/*neu* between human and murine is unexplained. Nevertheless, these results suggest that c-*erbB2* has a part in the development and/or progression of malignancies.

Transgenic mice present a useful system to assess the role of an oncogene in tumorigenesis of normal cells *in vivo*. With these mice it has been revealed that different oncogenes penetrate differentially into different types of cells (Stewart *et al.*, 1984; Adams *et al.*, 1985; Hanahan, 1985; Ornitz *et al.*, 1985, 1987; Leder *et al.*, 1986; Andres *et al.*, 1987; Mahon *et al.*, 1987; Sinn *et al.*, 1987; Suda *et al.*, 1987, 1988; Schoenberger *et al.*, 1988). Abnormal expression of some trans-oncogenic genes was compatible with normal development and differentiation, while other oncogenic genes were suppressed in normal cells. In most cases, tumors developed stochastically and clonally, corroborating the multistep nature of tumorigenesis. On the other hand, the mutant *neu* oncogene was proposed to induce malignant transformation of mammary epithelial cells in a single step when introduced under the control of mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) (Muller *et al.*, 1988).

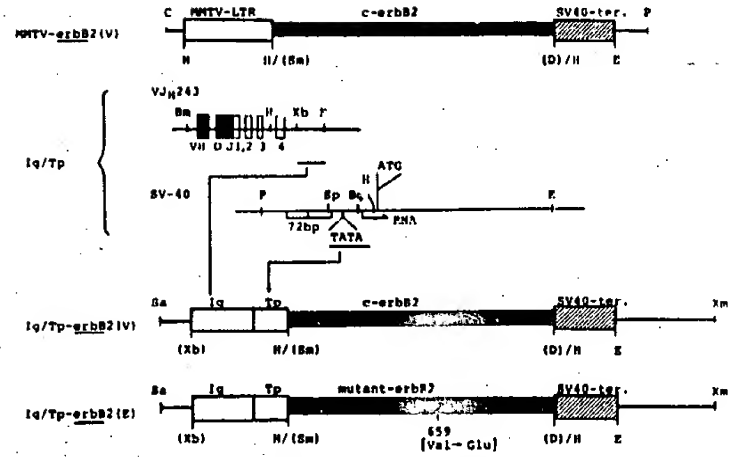

Fig. 1. Schematic representation of the trans-c-*erbB2* genes. Filled boxes represent c-*erbB2* cDNA, *erbB2*(V) or the cDNA in which the amino acid change is artificially introduced at position 659 from valine to glutamic acid *erbB2*(E). Hatched boxes indicate the termination signal from the SV40 early gene. Stippled boxes denote the regulatory unit, either MMTV-LTR or the joint fragment between mouse immunoglobulin enhancer and SV40 early gene promoter (Ig/Tp). Relevant restriction endonuclease sites are indicated. C, *Cla*I; H, *Hind*III; Sm, *Sma*I; D, *Dra*I; E, *Eco*RI; P, *Pst*II; Bm, *Bam*HI; Xb, *Xba*I; Sp, *Sph*I; Bg, *Bgl*I; Sa, *Sal*I; Xm, *Xma*I.

In this study, we first examined whether c-*erbB2*, when expressed at an elevated level, is oncogenic to unique cell types *in vivo*, by introducing normal c-*erbB2* into mice under the control of MMTV-LTR. A variety of tumors including adenocarcinomas developed after a relatively long latency; included among these were pre-B cell lymphomas. We then examined the role of c-*erbB2* in cell malignancy by generating mice harboring either normal or mutant c-*erbB2* under the control of immunoglobulin enhancer−SV40 promoter. With normal c-*erbB2*, pre-B cell lymphomas developed at 6−11 months, while with the mutant c-*erbB2* they develolped within a few months after birth. The lymphomas induced were monoclonal, unlike the polyclonal appearance of mammary adenocarcinomas induced by the mutant *neu* (Muller *et al.*, 1988).

Results

A variety of tumors with MMTV−erbB2(V)

In order to examine the oncogenicity of c-*erbB2* in a variety of tissues, a hybrid DNA composed of the MMTV-LTR and a cDNA encoding the c-*erbB2* protein (Figure 1) was constructed and microinjected into mouse zygotes. MMTV-LTR has been reported to direct the expression of transgenes not only in the mammary gland but also in a variety of tissues *in vivo* (Leder *et al.*, 1986; Choi *et al.*, 1987; Stewart *et al.*, 1988). The hybrid gene is designated MMTV−*erbB2*(V) and was confirmed to yield the correct transcript with glucocorticoid inducibility in NIH3T3 cells *in vitro* (data not shown).

In total, 205 zygotes obtained from the mating of F1(BL6 × SJL) × F1(BL6 × SJL) mice were microinjected and transferred into the oviducts of pseudopregnant CD1 females; 25 pups were born, five of which (20%) harbored the intact MMTV−*erbB2*(V) hybrid gene. One of these founder mice had insertions of the transgene at two separate unlinked sites and yielded two transgenic lines. In all of these transgenic lines (Merb1−6), the transgene was passed to the offspring in a Mendelian fashion.

A variety of tumors was induced in three (Merb1−3) of the six transgenic lines established, and further examinations focused on these lines. Some of the transgenic mice collapsed, suggesting a disorder of the respiratory system. Autopsies and histological analyses revealed the development of adenocarcinomas of lung in these animals (Figure 2A). The tumors were usually mutlicentric with some foci growing diffusely and some having typical papillary architecture. Other mice developed exophthalmia, which was confirmed to be caused secondarily by adenocarcinomas and/or hypertrophy of Harderian glands (Figure 2B). In most of these mice the symptoms were observed bilaterally, and histological features were highly heterologous, being partly hyperplastic and partly cancerous. These features suggest that the abnormal proliferation of these cells might also have been polyclonal in origin.

Although the occurrence of these adenocarcinomas of lung and Harderian gland was most common, lymphomas of T, B and non T, non B origin also developed in the Merb(V) lines. The most common type of T lymphoma was Thy1.2 positive but CD4 and CD8 double negative (Figure 3) with T cell receptor gene rearrangment, and B lymphomas were either B220 single positive or B220 and μ double positive (Figure 3) with immunoglobulin heavy chain (Ig_H) gene rearrangement. Histologically, the lymphomas were homogeneous, being composed of small blastic cells (Figure 2C and D), and the unique rearrangmenet pattern of T cell receptor or immunoglobulin heavy chain gene confirmed their clonal origin (data not shown). Non T, non B lymphoma cells, classified as such by their B220 and Thy1.2 negative nature (Figure 3) and germ line configuration of T cell receptor and immunoglobulin genes, were heterologous with large, oval- or spindle-shaped nuclei (Figure 2E). All these lymphomas had prominent mitotic figures and almost always infiltrated into non-lymphoid tissues. In addition to these tumors, hepatoma (Figure 2F) and leiomyosarcoma of the uterus (Figure 2G) developed as a single nodule in Merb(V) lines of mice in low frequency. The incidence is given in the pedigree analysis on the best-characterized Merb(V)2 line (Figure 4). The onset of tumors was relatively late and some mice were apparently healthy with no histological indication of lesions. Since no mammary adenocarcinomas developed in any of these transgenic mice, 12 females from Merb(V)1,

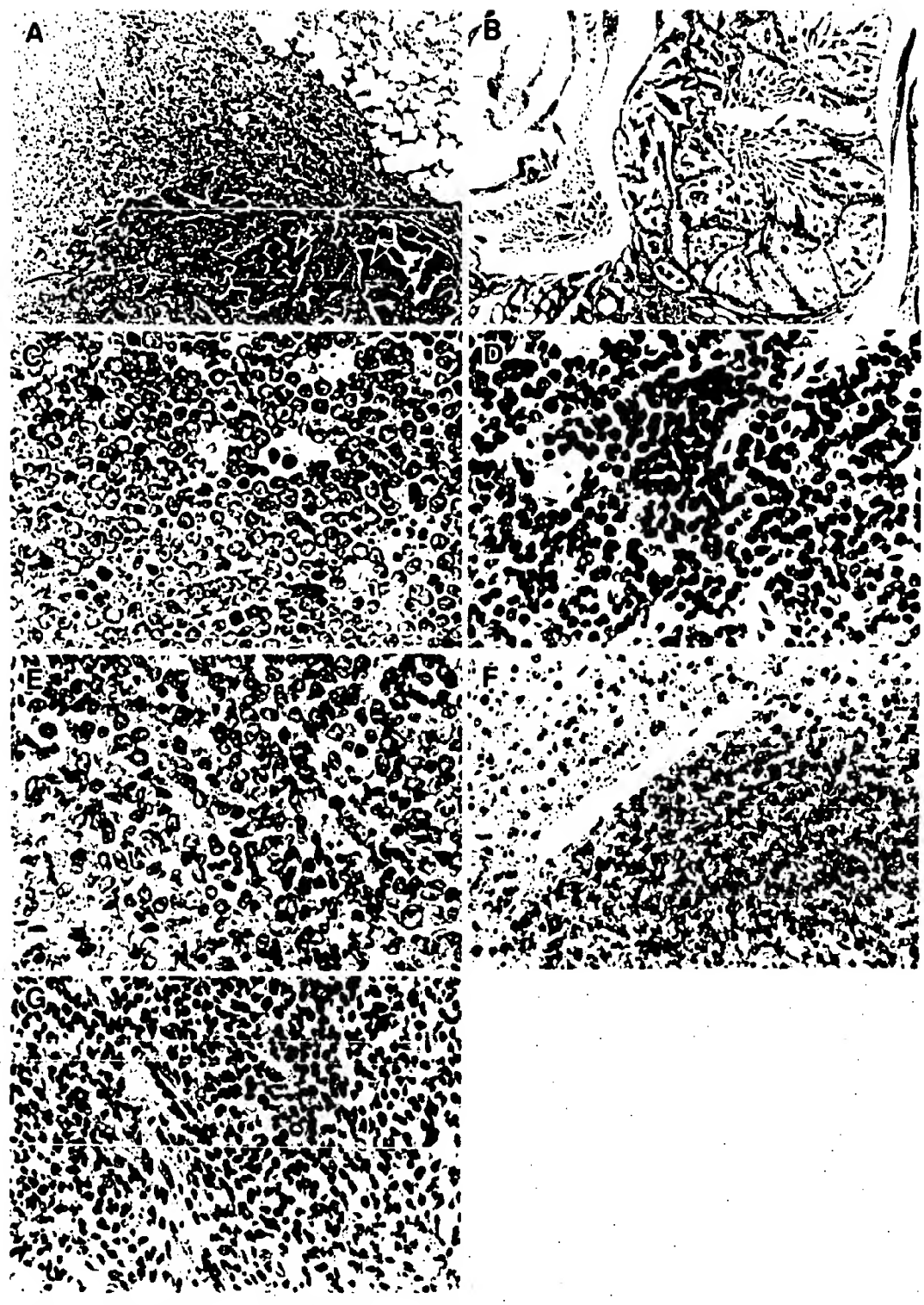

Fig. 2. Typical features of tumors induced with c-*erbB2* under the regulatory unit of MMTV-LTR. (A) Adenocarcinoma of the lung with medullary (arrows) and papillary (arrowheads) parts; (B) well-differentiated adenocarcinoma of Harderian gland. Optic nerve and retina are indicated by arrows; (C) T lymphoma cells with prominent nucleoli and with active mitotic figures; (D) B cell, lymphoma with active vascularization and mesenchymal trabeculae; (E) non B-non T lymphoma. Mitotic figures were abnormally high among cells with oval- and/or spindle-shaped nuclei; (F) a large nodule of hepatoma juxtaposed to normal hepatocyte; (G) leiomyosarcoma of the uterus. Small tumor cells were actively infiltrating into smooth muscle tissues.

2 and 3 lineages of various generations, which were triparons or more were sacrificed 2 months after their last pregnancy. No abnormality was observed in their mammary glands.

Adenocarcinomas of lung and Harderian glands and lymphomas of T and B cell origin are not common in laboratory mice and have never developed in our 65 age-

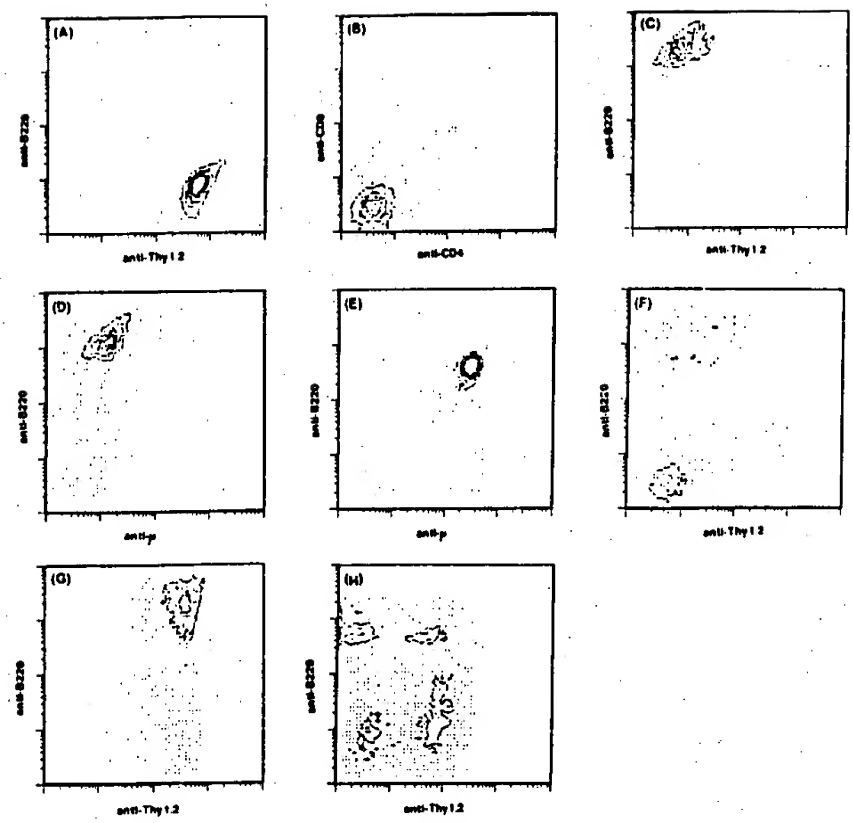

Fig. 3. Cell lineage analysis of lymphomas by flow cytofluorometry. Typical examples are shown: (A, B), T cell lymphoma developed in Merb(V)2-15 mouse; (C, D), pre-B lymphomas developed in Ierb(E)1-1 mouse; (E), B cell lymphomas developed in Merb(V)2-16 mouse showing similar staining pattern against Thy1.2 with panel (C); (F), non B-non T lymphomas developed in Merb(V)2-9 mouse; (G), unusual lymphomas developed in Ierb(E)1-3 mouse showing similar staining against CD4 and CD8 with panel (B); (H), unusual lymphomas developed in Ierb(V)1-33 mouse.

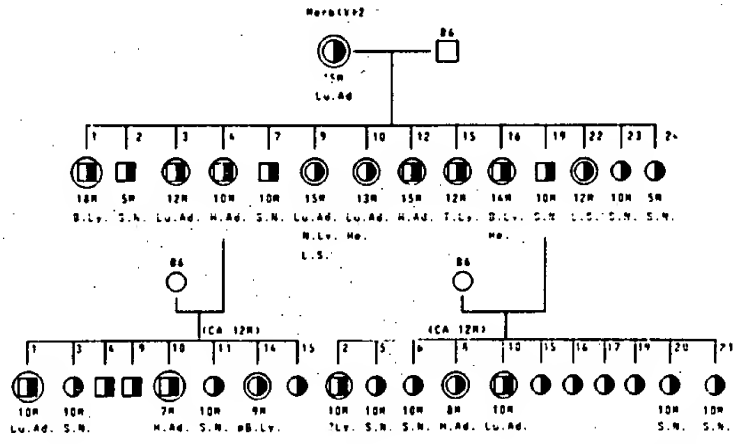

Fig. 4. Tumor incidence in Merb(V)2 lineage of mice. The tumor incidence is given for F0, F1 and F2 transgenic mice of Merb(V)2 lineage. Females are indicated by circles, males by squares. Only hemizygous transgenic mice obtained are shown with half-solid symbols. The outer circles denote tumor developments with the age at death (M, month after birth). The current age of apparently normal transgenic mice is given in each litter with the prefix CA. Lu.Ad., lung adenocarcinoma; H.Ad., Harderian gland adenocarcinoma; B.Ly., B lymphoma; pB.Ly., pre-B lymphoma; T.Ly., T lymphoma; N.Ly., non B-non T lymphoma; ?Ly, lymphoma of which cell surface properties could not be examined; L.S., leiomyosarcoma of uterus; He, hepatoma; S.N., sacrificed and normal.

matched non-transgenic control mice of the same genetic background.

Clonal pre-B cell lymphomas with Ig/Tp–erbB2(V)

To further dissect the process of malignant transformation by the c-*erbB2* gene, we planned to concentrate our study on the analysis of a specific tumor, B lymphoma, by introducing the c-*erbB2* cDNA into mice under the regulatory unit of immunoglobulin enhancer and SV40 early gene promoter (Ig/Tp) (Figure 1); clonality of tumors can be determined by rearrangement of the immunoglobulin gene in this type of tumor. It has been confirmed using the

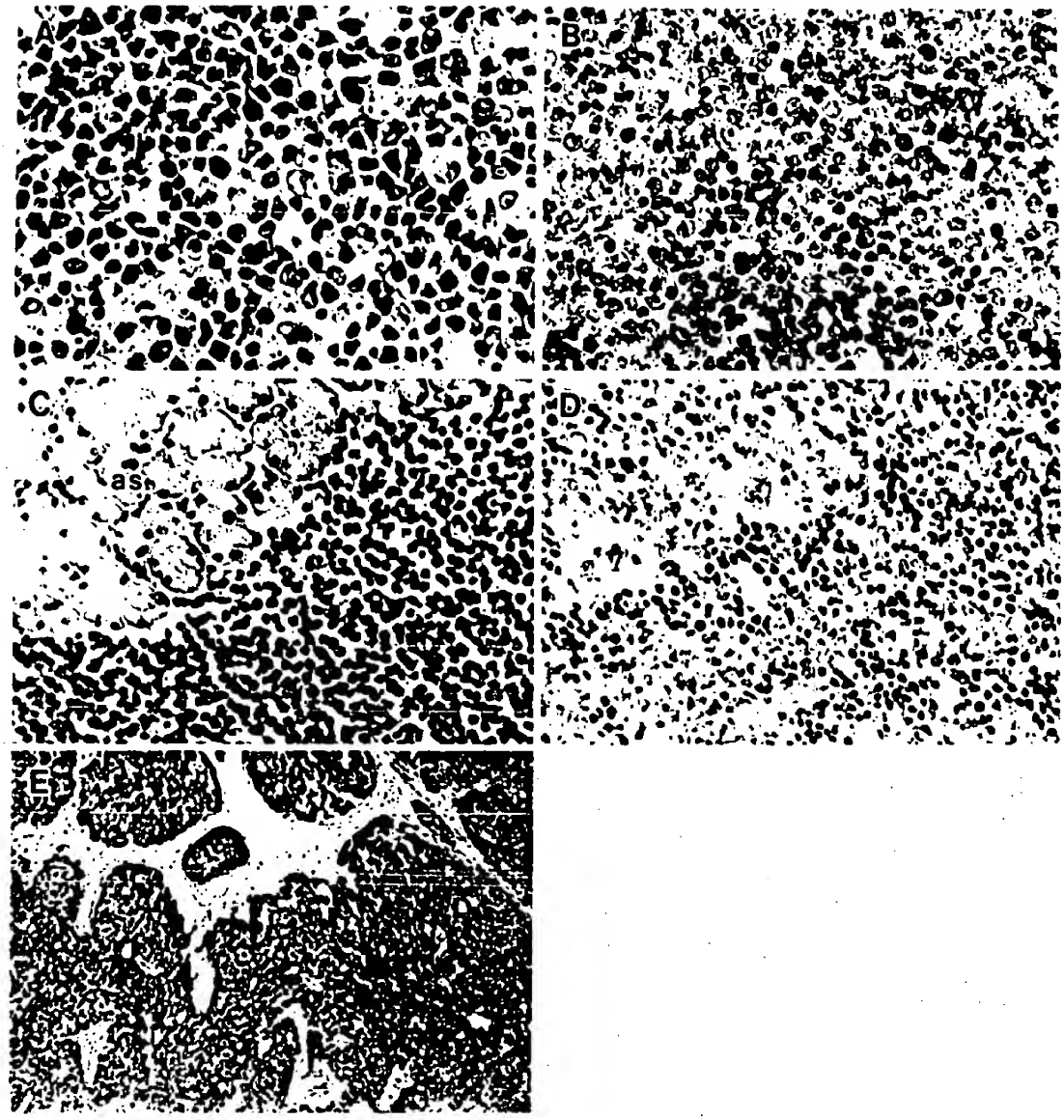

Fig. 5. Typical features of tumors induced with c-*erbB2* under the regulatory unit of Ig enhancer–SV40 early gene promoter. (A) Heterotypic pre-B lymphoma cells with heterotypical nuclei and with prominent mitotic figures, induced by the normal c-*erbB2*; (B) monotonous, immature pre-B lymphoma cells, induced by the mutant c-*erbB2*; (C) pancreas with the massive infiltration of lymphomas, induced by the mutant c-*erbB2* (as, pancreatic acinar cells); (D) moderately enlarged, but normal spleen in a pre-B lymphoma-bearing mouse with the mutant c-*erbB2*. Erythroblasts and megakaryocytes were prominent, indicating active hemopoiesis, with minimum tumorous lymphomas; (E) medullary-tubular adenocarcinoma of mammary gland with secretory ducts and secretion (arrows), developed in an offspring of Ierb(E)10 founder.

chloramphenicol acetyl transferase (CAT) gene (our unpublished result) and the SV40 large T gene (Suda *et al.*, 1987) as a marker gene in transgenic mice that this regulatory unit has strong transcriptional activity in lymphoid cells with some activity in heterologous cell types.

Normal c-*erbB2* cDNA was combined with this regulatory unit, yielding the hybrid gene Ig/Tp–*erbB2*(V) (Figure 1), and eight transgenic mice were obtained out of 32 pups born (25%). One of these founders was infertile, but seven (Ierb(V)1–7) yielded offspring and were established as lines harboring the intact DNA. Among these, Ierb(V)1 developed lymphomas at 10 months of age, while others remained healthy during the observation period of 15 months. All the lymph nodes of the Ierb(V)1 mouse swelled massively, and infiltration to the liver and lung was apparent macroscopically. The spleen was also greatly enlarged (1.3 g). Large cells with pale nuclei were peculiar among a variety of size of cells histologically having heterotypic nuclei (Figure 5A). The lymphoma cells showed prominent mitotic figures, actively infiltrated the liver, lung and other tissues, and were transplantable in nude mice. The cells were B220 positive and μ, Thy1.2 negative, and showed a unique Ig_H gene rearrangment pattern (Figuer 6) consistent with clonal pre-B cell lymphomas. In F1 transgenic offspring of Ierb(V)1 mouse, three out of 16 developed lymphomas by 11 months of age (Figure 7). Two of these three lymphomas were also pre-B in nature, but one showed unusual characteristics: the lymphoma cells were a composite of B220 and Thy1.2

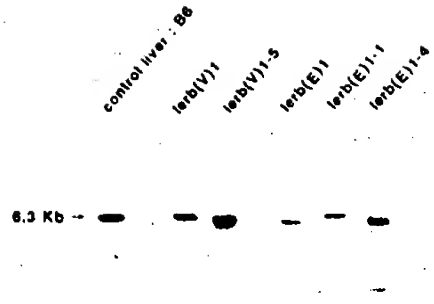

Fig. 6. Clonal nature of pre-B cell lymphomas induced by Ig/Tp-*erbB2*(V) and Ig/Tp-*erbB2*(E). Autoradiograms of restriction fragments of DNAs from lymphomas. DNAs of the tumorous lymph nodes from the mice indicated were digested with *Eco*RI and hybridized to a 450 bp *Hin*dIII–*Nae*I fragment containing JH sequences (Sakano *et al.*, 1980). The position of the fragment containing the germ-line allele of the immunoglobulin heavy chain gene is indicated by an arrow on the autoradiogram of the liver of a control mouse.

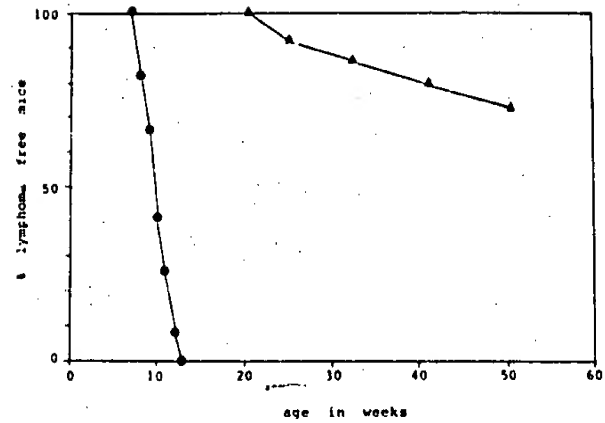

Fig. 7. Kinetics of lymphoma development. Comparison of the incidence of lymphoma formation between mice carrying either the Ig/Tp–*erbB2*(V) (▲) or the Ig/Tp–*erbB2*(E) (●). The kinetics are given on F0 and F1 mice of Ierb(V)1 for the former and of Ierb(E)1–4 for the latter.

double positive cells, double negative cells, B220 single positive cells and Thy1.2 single positive cells (Figure 3) with unique Ig_H gene rearrangement. The phenotype was retained in the transplanted tumors.

Neonatal onset of clonal pre-B cell lymphomas with the mutant c-erbB2

Possible increase in the oncogenicity of the c-*erbB2* gene by a mutation that alters the 659th amino acid in the transmembrane region from valine to glutamic acid was examined using the regulatory unit of immnoglobulin enhancer and SV40 promoter; the transgene was designated Ig/Tp–*erbB2*(E) (Figure 1). Ten transgenic mice harboring the intact transgene were obtained (Ierb(E)1–10) out of 52 pups born (10%). The frequency was somewhat lower (mice dying within a few days after delivery were not examined nor included in the number born in the present study). Four founders (Ierb(E)1–4) died of lymphomas within 2 months after birth. Because of neonatal onset of the lymphomas, no offspring could be obtained from Ierb(E)3 and Ierb(E)4 founders, but we were able to obtain eight and two transgenic offsprings from Ierb(E)1 and Ierb(E)2 founders, respectively, by *in vitro* fertilization. All the F1 offspring also developed lymphomas by 2.5 months. In these mice, all of the lymph nodes swelled massively with only moderate enlargement of the spleen (up to 0.3 g). Histological features showed a uniform population of small blastic cells with round nuclei and poor cytoplasm (Figure 5B). The blastic cells were actively infiltrating the pancreas (Figure 5C) and lung. A minimum number of malignant cells was present in the spleen, though they were moderately enlarged; histological features indicated active hematogenesis (Figure 5D). Seven out of the nine lymphomas examined were pre-B cell type as identified by cell surface markers (B220 positive, μ and Thy1.2 negative) (Figure 3), but two had unusual characteristics: B220 and Thy1.2 double positive and CD4 and CD8 double negative (Figure 3) with Ig_H gene rearrangement. All the lymphomas examined (five cases) were transplantable in nude mice and were thus malignant.

The rapid and uniform nature of kinetics by which the lymphomas developed in these lineages is shown in Figure 7, where the percentage of lymphoma-free mice is plotted as a function of age. Analyses of the Ig_H locus rearrangement pattern, however, showed distinctive fragments bearing a heavy-chain joining region (J_H) in equimolar yield (Figure 6). Polyclonal lymphomas should give smear bands because of random recombination. The result is precisely as expected for clonal lymphoid cells that have undergone diversity–joining region (DJ) or variable–diversity–joining region (VDJ) recombination at one or both Ig_H alleles, suggesting some additional events were necessary for the malignant transformation of the lymphoid cells.

The six founders, Ierb(E)5–10 had remained healthy and had no sign of disease when autopsied at 10 months old. Some offspring of the Ierb(E)10 founder, however, developed transplantable adenocarcinomas of the mammary gland (Figure 5E) and pre-B lymphomas; the incidence is under the examination, but is <5%. All the mice of Ierb(E)1–4 lineages with pre-B cell lymphomas had abnormal bone formation. This phenotype could be separated from the lymphoma-prone phenotype in transgenic mice generated from the Ierb(E)5–9 founders; these results will be reported elsewhere.

Expressions of trans-c-erbB2 genes and tumor developments

Figure 8 shows the Western blot analysis of the c-*erbB2* expression in tissues of Ierb(E)1 mouse. Since little expression of the endogenous c-*erbB2* product has been detected in various tissues of non-transgenic control mice (data not shown; cf. Mori *et al.*, 1989 and Figure 8B), the c-*erbB2* expression in transgenic mice is most probably due to the trans-c-*erbB2* gene. The gene was expressed intensively in neoplastic lymph nodes and the lymphomas transplanted in nude mice. It was also expressed intensively in the pancreas, possibly due to the enormous infiltration of neoplastic lymphoid cells in this tissue (Figure 5C). Similarly, the lung showed a moderate c-*erbB2* expression with a significant infiltration of the neoplastic cells. Although the spleen enlarged moderately, neoplastic cells were minimal, and active hematogenesis was suggested histo-

Fig. 8. Western blot analysis of trans-c-*erbB2* expression. Extracts from each tissue of (A) lymphoma-bearing Ierb(E)1 mouse, (B) apparently normal, precancerous Ierb(E)1-7 mouse and (C) lymphoma-bearing Ierb(V)1 mouse were subjected to Western analysis. The c-*erbB2* expression in MKN7 cells served as control.

logically (Figure 5D). This coincided well with the low expression of c-*erbB2* in this tissue. In tissues which had no infiltration of lymphoma cells, no c-*erbB2* expression could be detected. To confirm the correlation of c-*erbB2* expression with tumor development, an F1 offspring of Ierb(E)1, which was destined to develop the lymphoma as noted above (Figure 7) but had no sign of the disease yet, was killed at the age of 3 weeks in advance of lymphoma development. No expression could be detected in any tissues including lymphoid organs by either northern (data not shown) or Western blot analysis (Figure 8B). The same result was confirmed with two F2 offspring of the Ierb(E)1 founder 5 days after birth.

The c-*erbB2* protein was also expressed in tumorous tissues of Merb(V)2 and Ierb(V)1 mice. The level of the c-*erbB2* expression in lymphomas induced by the normal Ig/Tp−*erbB2*(V) was roughly the same as that in lymphomas induced by the mutant Ig/Tp−*erbB2*(E) when compared, using the expression in MKN7 cells as a standard (Figure 8C). However, the level of the c-*erbB2* protein in tumors induced by MMTV−*erbB2*(V) was less extensive (data not shown), which may coincide with the relatively late onset of the tumors in mice of the Merb(V) series. The normal c-*erbB2* gene was also not expressed in any normal tissues of young mice of the Ierb(V) or Merb(V) series which had no sign of disease.

NIH3T3 was not transformed with DNAs from lymphomas induced by c-erbB2

Strong oncogenicity of the mutant c-*erbB2* gene and relatively late and stochastic onset of the lymphomas with the normal c-*erbB2* gene may give rise to the question of whether the lymphomas are induced by the latter via mutational activation. As noted earlier, two mutations are necessary for the amino acid change at position 659 from valine to glutamic acid, and this activation would be quite a rare event. The possibility remains, however, that there are activational mutations at unknown sites. To examine this possibility, DNAs of the lymphomas induced with the normal c-*erbB2* gene were transfected into NIH3T3 cells. However, no transformed focus containing transforming c-*erbB2* was obtained, while DNAs of the lymphomas induced with the mutant c-*erbB2* yielded numerous foci (data not shown).

Discussion

The behavior of the normal and the mutant alleles of the c-*erbB2* oncogene in transgenic mice provides insight into the process of the malignant transformation induced by this gene. The present results strongly suggest that the normal c-*erbB2* gene is oncogenic in normal cells *in vivo* when expressed at high levels, consistent with results using NIH3T3 cells *in vitro* (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987). The tumor induction was observed not only with MMTV-LTR, but also with the regulatory unit composed of immunoglobulin enhancer and SV40 early gene promoter. The possibility that tumor induction with normal c-*erbB2* occurred via mutational activation is less likely because two nucleotide mutations are necessary for the amino acid change at position 659, and no active c-*erbB2* gene could be recovered from the tumors by NIH3T3 transfection assay.

Unexpectedly, adenocarcinomas were not induced in the mammary gland of any transgenic lines harboring normal c-*erbB2* under the control of MMTV-LTR, while the transforming allele of the c-*neu* did reportedly cause such induction (Muller *et al.*, 1988) and the mutant c-*erbB2* did, even under the control of Ig/Tp in some offspring of the Ierb(E)10 founder. Since the c-*erbB2* gene was expressed only in tumor tissues and not in any normal tissues including mammary glands, it is uncertain whether or not the normal c-*erbB2* products are oncogenic in the epithelial cells of the breast. It is always possible that accidental changes in MMTV-LTR during construction may result in loss of the capability to direct efficient expression in the mammary gland. However, under control of MMTV-LTR, SV40 large T gene is reported to induce adenocarcinomas frequently in lung and kidney, but rarely in mammary gland (Choi *et al.*, 1987). In any event, the most frequent type of tumor induced by normal c-*erbB2* under MMTV-LTR was the adenocarcinoma, which is consistent with the idea that this gene contributes to the development and/or progression of adenocarcinomas (Yokota *et al.*, 1986, 1988; Masuda *et al.*, 1987; Van de Vijver *et al.*, 1987; Ali *et al.*, 1988; Juerin *et al.*, 1988; Tal *et al.*, 1988). In addition, lymphomas of a variety of cell types were also induced by MMTV−*erbB2*(V). This is not surprising since MMTV-LTR reportedly directed the expression of transgenes in lymphoid tissues (Leder *et al.*, 1986; Choi *et al.*, 1987, 1988; Stewart *et al.*, 1988). The induction of pre-B cell lymphomas with the normal c-*erbB2* gene was also confirmed by the Ig/Tp regulatory unit which has strong transcriptional activity in B lineage of cells. Although the c-*erbB2* gene is naturally dormant in lymphoid cells (Gullick *et al.*, 1987; Kokai *et al.*, 1987; Mori *et al.*, 1989), and its contribution to lymphomas has not yet been suggested in human cancer (Yokota *et al.*, 1986, 1988; Masuda *et al.*, 1987; Van de

Vijver *et al.*, 1987; Ali *et al.*, 1988; Juerin *et al.*, 1988; Tal *et al.*, 1988), our present data suggested that c-*erbB2* products were oncogenic not only in epithelial cells, but also in lymphoid and a variety of other cells.

Some of the lymphomas induced by the Ig/Tp regulatory unit had unusual characteristics such as B220 and Thy1.2 double positive or a mixture of cells with heterogeneous cell surface properties, with unique Ig_H gene rearrangement. Such lymphomas may be explained as tumor-induced 'lineage infidelity' (McCulloch, 1983) or as multiphenotypic cells derived from rare normal cells (Greaves *et al.*, 1986). Characterization of these lymphomas is subject to further examination.

The kinetics of lymphoma induction with normal c-*erbB2* is similar to our previous experience with transgenic lines bearing Ig/Tp-*myc* (Suda *et al.*, 1987); the lymphoma developed only in certain transgenic offspring with variable onset, while others remained normal for >1 year. The stochastic occurrence of clonal tumors after a long latency period is reminiscent of transgenic mouse lines carrying other oncogenes such as *myc*, *ras*, *fos*, *int*-1 and *pim*-1 under various regulatory units (Stewart *et al.*, 1984; Adams *et al.*, 1985; Leder *et al.*, 1986; Andres *et al.*, 1987; Ruther *et al.*, 1987; Schoenenberger *et al.*, 1988; Tsukamoto *et al.*, 1988; Lohuizen *et al.*, 1989), and corroborates the notion that some additional events are necessary to induce tumors with these genes. In contrast, the lymphomas were induced with the mutant c-*erbB2* in rapid and uniform kinetics similar to lung adenocarcinomas induced by the active c-Ha-*ras* under the same Ig/Tp regulatory unit (Suda *et al.*, 1987) and to mammary gland adenocarcinomas induced by the active *neu* with MMTV-LTR (Muller *et al.*, 1988). Histologically, the mammary adenocarcinomas induced by the active *neu* seemed to arise from the entire mammary gland epithelium, and lung adenocarcinomas induced by the active *ras* were multicentric but did not arise from all lung epithelial cells. Some question remains of their polyclonality since no genetic or biochemical marker exists in these types of cells. However, pre-B cell lymphomas induced by the mutant c-*erbB2* were monoclonal judging by immunoglobulin heavy chain rearrangement. Apparently mutant c-*erbB2* required additional events to accomplish malignant transformation of the lymphoid cells. There is a possibility that the Ha-MuSV-derived 600 bp sequences present in Muller *et al.*'s construct (Ellis *et al.*, 1980; Huang *et al.*, 1981; Muller *et al.*, 1988) have some relevance in mammary tumor development (Gruss *et al.*, 1981). It is also possible that the c-*erbB2*/*neu* gene penetrates differentially into mammary epithelial and lymphoid cells. Recently, Bouchard *et al.* (1989) reported stochastic induction of mammary tumors by activated *neu* with MMTV-LTR.

We previously observed that under the same Ig/Tp regulatory unit *myc* and *ras* genes were expressed extensively not only in tumor tissues, but also in normal tissues, and their expressions were compatible with normal differentiation of embryonic stem cells *in vivo* (Suda *et al.*, 1987, 1988). Similar conclusions were reported by many groups under various transcriptional regulatory units (Leder *et al.*, 1986; Andres *et al.*, 1987; Ruther *et al.*, 1987; Sinn *et al.*, 1987; Schoenenberger *et al.*, 1988; Tsukamoto *et al.*, 1988; Lohuizen *et al.*, 1989). The pattern of c-*erbB2* (not only normal but also mutant c-*erbB2*) expression is in sharp contrast to this; the gene was expressed intensively in tumor tissues and not in normal tissues, though the possibility of an undetectable low level expression in normal cells cannot be ruled out. Nevertheless, the level of its expression in tumor cells was comparable with that in MKN7 cells in which the c-*erbB2* gene is amplified and over-expressed (Fukushige *et al.*, 1986). We observed a similar situation with the SV40 T gene under the same Ig/Tp regulatory unit, as did Ornitz *et al.*, under elastase I regulatory unit (Suda *et al.*, 1987, 1988; Ornitz *et al.*, 1985, 1987). In mice harboring the active *neu* with MMTV-LTR (Muller *et al.*, 1988), the *neu* gene is also likely to be suppressed throughout the course of development and differentiation, and an increase in its expression beyond the threshold would be necessary to induce the transformation, judging from Muller *et al.*'s observation of their TG.NK line of transgenic mice. Thus, the active c-*erbB2*/*neu* is in contrast to the active *ras* with which modest amounts of the products are sufficient for transformation in conjunction with unknown secondary events (Quaife *et al.*, 1987; Suda *et al.*, 1987). Desuppression or induction of enhanced expression of the active *neu* under MMTV-LTR seemed to occur in all mammary epithelial cells following sexual maturation, possibly through a hormonal effect, but that of c-*erbB2* under Ig/Tp regulatory unit occurred in only a few lymphoid cells. Whatever the mechanism regulating expression of these transgenes differentially *in vivo*, the observation with c-*erbB2* is consistent with the idea that tumor induction with this gene is accompanied by a high level of its expression.

The secondary event(s) necessary to induce lymphoid tumors with active c-*erbB2* might thus simply be the gene's expression beyond the threshold. This may occur at the same frequency as the normal c-*erbB2*-transgene. No difference exists between these transgenes except two nucleotide changes. The necessity of additional event with the normal c-*erbB2* may explain the stochastic occurrence and later onset of the lymphomas. However, though preliminarily, a unique chromosomal change commonly observed in pre-B cell lymphomas induced with *myc* (M.Oshimura, Y.Suda and S.Aizawa, to be published), was observed in some lymphomas induced with the mutant *erbB2*. Thus, whether or not a high level of expression of the mutant c-*erbB2* is alone adequate for malignant transformation of pre-B cells requires further examination.

Materials and methods

Construction of hybrid genes

A plasmid containing the full-length *erbB*-2 cDNA was constructed by recombining the two cDNA clones pCER204 and pcER235 (Yamamoto *et al.*, 1986). The resulting plasmid pCERSH22 was then partially cleaved by *Sma*I and *Dra*I, and ligated with *Hin*dIII linker. The fragment was replaced with the *dhfr* gene in pSV2 (Lee *et al.*, 1981), yielding pSV2*erbB2*(V). In order to construct the pMMTV-*erbB2*(V) recombinant, the pMDSG plasmid (Ringold *et al.*, 1981) which contains MMTV-LTR was partially digested with *Hin*dIII and *Pst*I. Then, the fragment containing the MMTV-LTR sequences was replaced with the corresponding sequences of pSV2*erbB*-2(V) after partial *Hin*dIII and *Pst*I digestion. In order to construct the plg/Tp-*erbB2*(V) and plg/Tp-*erbB2*(E) recombinants, the plasmid plg/Tp-*neo* was first obtained by ligating the *Ace*I–*Hin*dIII fragment of the plasmid plg/Tp-*T* containing immunoglobulin enhancer and SV40 promoter (Suda *et al.*, 1988) with the *Hin*dIII–*Ace*I fragment of the plasmid pSV2*neo* (Southern and Berg, 1982). The plasmid plg/Tp-*neo* was then cleaved with *Hin*dIII and *Sma*I to remove *neo*-derived sequences and was ligated with *Hin*dIII linker. The fragment was ligated with *Hin*dIII fragment of the plasmid pSV2*erbB2*(V) or the plasmid pSV2*erbB2*(E) containing the normal c-*erbB2* cDNA or the mutant c-*erbB2* cDNA respectively. Details of the construction

of pSV2*erbB2*(V) and pSV2*erbB2*(E) will be reported elsewhere (Y.Yamada, K.Toyoshima and T.Yamamoto, to be published).

Production of transgenic mice

pMMTV-*erbB2*(V) was digested wth *Cla*I and *Pvu*I, and plg/Tp-*erbB2*(V) and plg/Tp-*erbB2*(E) with *Sal*I and *Xmn*I. The fragments were separated by electrophoresis in 1% agarose gel, solubilized with NaI solution, adsorbed to a glass powder, solubilized and diluted with an injection buffer (10 mM Tris–HCl pH 7.4, 0.25 mM EDTA) to a final concentration of 1–2 μg/ml. Fertilized eggs were obtained from superovulating F1 females (4–5 weeks) by mating with F1 males. The F1 mice were prepared by crosses between C57Bl/6 females (Shizuoka Farms) and SJL males (Jackson Lab.). The DNA solution was injected into the male pronucleus as previously described (Suda *et al.*, 1987). Following microinjections, the eggs were kept for 2 h at 37°C in M16 medium and viable eggs identified by gross morphology were transferred to the oviducts of pseudopregnant CD-1 mice (Charles River, Japan).

Identification of transgenic mice

High mol. wt DNAs extracted from ~1 cm tail sections of newborn mice were digested with *Eco*RI and the digests were electrophoresed on 1% agarose gels. The fractioned DNAs were then transferred to nitrocellulose paper (Southern, 1975). Presence of the transgenes was probed by hybridizing the filters with the c-*erbB2* cDNA radiolabeled with [^{32}P]dCTP by nick translation as previously described (Suda *et al.*, 1987).

RNA analyses

Total RNAs were extracted from tissues by the guanidine thiocyanate method (Chirgwin *et al.*, 1979) in conjunction with ultracentrifugation through a cesium chloride cushion (Glisin *et al.*, 1974). The RNAs were dissolved in 0.2 M Na-acetate and precipitated with ethanol. Poly(A)$^+$ RNAs were isolated from total RNAs on an oligo(dT) cellulose column and electrophoresed on a 1% agarose–formaldehyde gel. The fractionated RNAs were transferred to nitrocellulose filters (Thomas, 1980). The filters were then hybridized with the ^{32}P-labeled c-*erbB2* cDNA probes in 50% formamide, 10 × Denhardt's, 0.05 M sodium phosphate (pH 6.5) and 5 × SSC at 42°C for 15 h. After hybridization, the filters were washed in 0.2 × SSC, 0.1% SDS at 50°C for 2 h.

Western blotting

Tissues were homogenized in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride, 2 mM aprotinin) at 4°C and the homogenates were centrifuged for 1 h at 30 000 r.p.m. The soluble extracts (100 μg total protein) were subjected to 7.5% SDS–PAGE and then electrophoretically transferred to a nitrocellulose filter. The nitrocellulose paper was blocked in TBS (100 mM Tris, 1.5 M NaCl) containing 2% skimmed milk overnight, followed by incubation in a buffer composed of 0.05% Tween 20, 2% skimmed milk, TBS containing a 1:1000 dilution of anti-c-*erbB2* polyclonal antibody for 1 h at 4°C. The filter was washed with TBS and was reacted with alkaline phosphatase-conjugated anti-rabbit IgG. It was then extensively washed with 0.05% Tween 20 in TBS, dried and developed with AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing the substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

Histologic analyses

Complete autopsies were performed whenever possible and organs were examined both grossly and histologically. For the latter, tissues were fixed with 4% formaldehyde in PBS and fixed tissues were embedded in paraffin. The paraffin blocks were sectioned at 4 μm thickness and the sections routinely stained with hematoxylin and eosin.

Cell lineage analyses of lymphoid tumors

Tumorous tissues were finely minced and dispersed in PBS containing 10% serum by rubbing with two pieces of ground slide glass. Singly dispersed cells were collected by passing them through 100 μm mesh. The cells were stained on ice for 20 min with antibodies for surface markers of each subset of lymphoid cells in SM solution (4% fetal calf serum, 0.1% NaN_3 and 1 μg/ml propidium iodide in PBS). Staining with rabbit anti-mouse Thy1.2 antibody, rabbit anti-mouse μ antibody, rabbit anti-mouse Mac1 antibody and rabbit anti-mouse CD8 antibody was performed directly with fluorescent isothiocyanate (FITC)-labeled antibody. The staining with rabbit anti-mouse B220 antibody, rabbit anti-mouse AA4 antibody and rabbit anti-mouse CD4 antibody was performed with biotinylated antibody and phycoerythrin-labeled avidin. Stained cells were anlayzed flow cytofluorometrically with a FACS IV (Becton Dickinson & Co.). Intact cells were demarcated from dead cells and debris using forward and sideways light scatter. Five thousand particles were analyzed for single color fluorescence as noted (Nakauchi *et al.*, 1987).

In vivo transplantation of tumors

Non-lymphoid tumors chopped into small pieces were grafted and passaged under the skin of nu/nu mice. Lymphoid tumors were singly dispersed in PBS containing 10% serum by rubbing chopped pieces with two pieces of ground slide glass. The dispersed cells were transplanted and passaged in the abdominal cavity and/or under the skin of nu/nu mice. The mice were purchased from Charles River Japan (CD1-nu).

NIH3T3 transfection assay

High mol. wt DNAs prepared from tumor tissues were sheared once through a 20 gauge needle. Aliquots of shared DNAs were ethanol-precipitated and resuspended in 2.5 ml of transfection buffer [0.7 mM $Na_2HPO_4 \cdot 7H_2O$, 21 mM HEPES, 0.145 M NaCl (pH 7.0)]. Transfection to NIH3T3 cells was performed by the calcium phosphate precipitation technique of Graham and van der Eb (1973), as modified by Anderson *et al.* (1979). One hundred and twenty five μl of 2.5 M $CaCl_2$ was added to the above solution and mixed immediately by vortexing. As soon as fine bluish precipitates were apparent, 1.25 ml of the solution was applied onto 7×10^5 NIH3T3 cells in 10 ml of Dulbecco's modified Eagle's medium containing 10% calf serum. The calcium phosphate–DNA precipitates were removed 4 h after the initial application by placing in a fresh culture medium. The medium was changed twice a week and the transformed colonies were identified after 14–21 days of culture.

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